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Expression and functional evaluation of transient receptor potential channel 4 in bovine corneal endothelial cells

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Abstract

We previously found that activation of purinergic receptors mobilizes Ca^{2+} and enhances bicarbonate transport in bovine corneal endothelial cells (BCEC). Since transient receptor potential channel 4 (TRPC) has been reported to be a candidate for capacitative calcium entry (CCE) and receptor operated calcium entry (ROC), we examined the expression of TRPC4 and evaluated the potential involvement of TRPC4 in CCE or ROC in BCEC.

The C-terminus of TRPC4 was fused into the glutathione S-transferase (GST) expression vector. The fusion protein GST-TRPC4c was induced in bacteria and purified by affinity chromatography. An antibody was raised in rabbit by using the purified GST-TRPC4c antigen. In Western blotting, the TRPC4 antibody recognized the fusion protein while the pre-immune IgG did not. The TRPC4 antibody recognized a band at around 80 kD for membrane proteins from both the fresh and cultured BCEC. The pre-immune IgG could not detect bands at the same size. Incubation with the TRPC4c antigen abolished the 80 kD band. Immunofluorescence using the TRPC4 antibody stained both fresh and cultured BCEC, while pre-immune IgG did not. RNAi knocked down the expression of TRPC4 in cultured BCEC. Ca^{2+} entry induced by the purinergic receptor agonist ATP, was increased in TRPC4-siRNA transfected cells compared with the scrambled siRNA control, while Ca^{2+} entry induced by store depletion through blocking the endoplasmic reticulum Ca^{2+} pump, did not differ between the siRNA and scrambled siRNA-treated cells. Taken together, these results show that TRPC4 protein is expressed in the bovine corneal endothelial cells and may be a negative regulator in ROC stimulated by purinergic activation, but not by store depletion itself.

Keywords

cornea; endothelium; bovine; TRPC4; antibody generation; capacitative calcium entry; receptor operated calcium entry

1. Introduction

The corneal endothelium is a thin monolayer of cells covering the posterior surface of the cornea. The primary function of the corneal endothelium is to maintain corneal transparency through active transport of ions and fluid from corneal stroma to the anterior chamber of the eye (Bonanno, 2003; Fischbarg, 2003; Fischbarg and Maurice, 2004). The corneal endothelial fluid transport is dependent on the presence of HCO_3^- (Riley et al., 1995). We have previously shown that basolateral HCO_3^- permeability is significantly higher than apical (Bonanno et al., 1999). Thus the rate-limiting step in transendothelial HCO_3^- transport is at the apical membrane. Recently, we found that apical HCO_3^- permeability can be enhanced by increasing $[\text{Ca}^{2+}]_i$ with the purinergic agonist ATP (Zhang et al., 2002).

G_q -protein coupled receptor (GPCR) activation (e.g. P2Y) involves generation of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol by phospholipase C (Berridge, 2000). The binding of IP_3 to IP_3 receptors (IP_3R), which are located in the membrane of the endoplasmic reticulum (ER), activates the intrinsic Ca^{2+} channel leading to release of Ca^{2+} from the ER (the Ca^{2+} stores) into the cytosol. The release of Ca^{2+} is closely followed by the entry of extracellular Ca^{2+} into the cytoplasm across the plasma membrane, a process called 'capacitative calcium entry' (CCE) or 'store-operated calcium entry' (Bolotina, 2004; Penner and Fleig, 2004; Putney, 1986, 2004; Venkatachalam et al., 2002). CCE can be alternatively induced by emptying the Ca^{2+} store with the use of inhibitors of the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA), which actively transports Ca^{2+} from the cytosol into the ER.

In addition to CCE, Ca^{2+} also enters the cell through store-independent pathways, which are termed receptor activated Ca^{2+} entry, receptor induced Ca^{2+} entry, or receptor operated Ca^{2+} entry (ROC) (Barritt, 1999; Berridge, 2000; Zitt et al., 2002; Shuttleworth, 2004; Gill and Patterson, 2004). To our knowledge, the molecular identity of ROC has not been determined. ROC may be activated by different second mediators such as IP_3 (Kuno and Gardner, 1987; Mozhayeva et al., 1991; Restrepo et al., 1990), IP_4 (Luckhoff and Clapham, 1992), arachidonic acid (Mignen and Shuttleworth, 2001; Mignen et al., 2001), diacylglycerol (Hofmann, 1999), and the trimeric G proteins (Krautwurst et al., 1992), which may be generated at the same time CCE is induced. In addition, ROC activity may also be modulated by protein kinase C (Venkatachalam et al., 2003).

In BCEC, apical HCO_3^- permeability can be enhanced by increasing $[\text{Ca}^{2+}]_i$ via either activation of purinergic receptors or inhibition of SERCA, which can induce both intracellular Ca^{2+} release and CCE (Zhang et al., 2002). The presence of purinergic P2Y receptors in BCEC has been reported (Srinivas et al., 1998, 2002; Cha et al., 2000). CCE and ROC have been demonstrated in corneal endothelial cells by multiple studies (Crawford et al., 1992, 1993; Srinivas et al., 1998; Xie et al., 2002; Cha et al., 2000). We also found that a Ca^{2+} influx factor from P450 metabolism and secretion-like coupling mechanisms play roles in CCE in corneal endothelial cells (Xie et al., 2002). However, the molecular identity for Ca^{2+} entry induced by purinergic stimulation or CCE is not clear. Since transient receptor potential channel 4 (TRPC4) was reported to be a putative Ca^{2+} channel for CCE (Freichel et al., 2001; Philipp et al., 2000; Tiruppathi et al., 2002; Wang et al., 2004) or ROC

(Schaefer et al., 2000; Wu et al., 2002), we examined if TRPC4 is expressed in BCEC and plays a role in regulating CCE or ROC. We generated an antibody against TRPC4 and identified the expression of TRPC4 by Western blotting and immunostaining. In order to characterize the function of TRPC4 in BCEC, we evaluated Ca^{2+} entry by knocking down the expression of TRPC4 with the RNA interference technique.

2. Materials and methods

Cell culture

Bovine corneal endothelial cells were cultured to confluence on glass coverslips (Bonanno and Giasson, 1992). Briefly, primary cultures from fresh cow eyes were established in T-25 flasks in 3 ml of Dulbecco's modified Eagle's medium, 10% bovine calf serum with antibiotic antimycotic agents (100 units ml^{-1} penicillin, 100 $\mu\text{g ml}^{-1}$ streptomycin, and 0.25 $\mu\text{g ml}^{-1}$ fungizone); gassed with 5% CO_2 –95% air at 37°C; and fed every 2–3 days. These cells were subcultured to coverslips. The cells were transferred to 0.5% serum with Dulbecco's modified Eagle's medium for at least 12 hr before experiments.

Solutions and chemicals

The composition of Ca^{2+} -rich Ringer solution was 157.7 mM Na^+ , 4 mM K^+ , 0.61 mM Mg^{2+} , 1.4 mM Ca^{2+} , 151.02 mM Cl^- , 1 mM HPO_4^{2-} , 10 mM HEPES, 12.7 mM gluconate $^-$, and 5 mM glucose. Ca^{2+} -free solution was composed of 157.7 mM Na^+ , 4 mM K^+ , 0.61 mM Mg^{2+} , 148.22 mM Cl^- , 1 mM HPO_4^{2-} , 10 mM HEPES, 12.7 mM gluconate $^-$, 0.5 mM EGTA, and 5 mM glucose. For all solutions, the osmolarity was adjusted to 300 ± 5 mOsm with sucrose, and the pH was adjusted to 7.4.

Fura-2 acetoxymethyl ester was obtained from Molecular Probes (Eugene, OR). Cell culture supplies were obtained from Invitrogen. All other chemicals were obtained from Sigma. Stock solutions of the above chemicals were stored desiccated at -20°C .

2.1. RT-PCR

RT-PCR was performed using 0.4 μg of total RNA from cultured BCEC. The RT step was carried out in a total volume of 20 μl using Superscript II reverse transcriptase according to the manufacturer's instructions (Invitrogen). The PCR reactions were carried out in a total volume of 50 μl using the high-fidelity Taq DNA polymerase according to the manufacturer's instructions (Roche, Indianapolis, IN). Thirty-five PCR cycles (95°C for 30 sec, 61°C for 30 sec, and 72°C for 30 sec) were performed in addition to a beginning denaturation at 95°C for 2 min and a final extension at 72°C for 10 min. PCR primers used for amplifying TRPC4 were described by Philipp et al. (2000). The expected product is 304 bp. The primers for GAPDH were described previously (Sun et al., 2004). The RT-PCR products were subject to 1.5% agarose gel electrophoresis and visualized by ultraviolet illumination. The band was cut and purified by Qiagen gel extraction kit (Qiagen, Valencia, CA). The purified PCR product was subcloned into the pcDNA3.1-V5-His-TOPO vector (Invitrogen). The PCR product was finally sequenced using the T7 primer (IUPUI DNA sequencing facility, Indianapolis, IN).

2.2. Generation of the anti-TRPC4 antibody

The C-terminal segment 755–975 aa was PCR amplified using the mouse cDNA as template (a kind gift of Dr Michael X. Zhu). The PCR product was cloned into pGEX-6P-3 (Amersham, Piscataway, NJ) so that the encoding amino acid sequences are downstream of the glutathione S-transferase (GST) protein. The resultant fusion protein is named GST-TRPC4c. Proteins were induced at 30°C by IPTG (0.25 mM). Bacteria were sonicated and centrifuged. GST-TRPC4c was soluble and purified by affinity chromatography using the glutathione sepharose fast flow agarose beads (Amersham, Piscataway, NJ). The purified GST-TRPC4c was injected into a New Zealand White rabbit (Covance, Philadelphia, PA). The resultant antiserum, anti-TRPC4c, was purified by affinity chromatography. Briefly, purified GST and GST-TRPC4c were covalently coupled to CNBR sepharose beads (Amersham Biosciences, Piscataway, NJ). The sera were first purified by protein A/G (Sigma–Aldrich, St Louis, MO) and then by the GST-CNBR beads to absorb anti-GST IgGs. GST-TRPC4c CNBR beads were then used to purify the remaining IgGs. Finally, purified antibodies were eluted with 0.2 M glycine (pH = 2.8) and neutralized with 1 M Tris–HCl pH 8.0, resulting in the anti-TRPC4c antibody. At the same time, the same volume of the pre-immune serum went through the same procedure, the resultant purified IgG fraction, named pre-immune IgG, was used as control.

2.3. Western blotting

Membrane proteins were extracted by ultracentrifugation as described previously (Xie et al., 2000). Briefly, bovine brain tissues or BCECs were homogenized in microsome preparation buffer (0.1 M Tris–Ac, 1 mM EDTA, 0.15 M KCl, pH 7.4) and centrifuged at 12 000 g 4°C for 20 min. The supernatant was subject to ultracentrifugation at 100 000 g and at 4°C for 1 hr. The resultant pellets were solubilized with sample buffer without dye (2% SDS, 0.5 M Tris–HCl 6.8, 20% glycerol).

Protein concentration was quantitated by Bio-rad protein assay reagents (Hercules, CA) and separated by 8% SDS-PAGE. Ten micrograms of proteins were loaded for each lane. The samples were then transferred to PVDF membranes, which were blocked with 5% non-fat dry milk in PBST for 1 hr at room temperature, incubated with primary antibodies (T4nAb, the anti-TRPC4c antibody, or the pre-immune IgG, 1:1000; β -actin, 1:10 000) containing 5% milk for an additional hour, washed with PBST, and then incubated with a secondary antibody at 1/10 000 dilution in PBST containing 5% milk. The T4nAb is a rabbit polyclonal antibody against the TRPC4 protein (a kind gift of Dr Michael X. Zhu) (Tang et al., 2000). The rabbit anti-HA antibody and the mouse monoclonal antibody against β -actin were from Sigma. The secondary antibodies were peroxidase-conjugated goat anti-rabbit or mouse IgG (Sigma, St Louis, MO) and the blots were detected with an enhanced chemiluminescence kit (ECL) as described by the manufacturer (Pierce, Rockford, IL).

2.4. Transfection

To test if the anti-TRPC4c antibody can recognize the TRPC4 protein, we transfected TRPC4 using the pcDNA3-TRPC4-HA expression construct (a kind gift of Dr Michael X. Zhu) into HEK 293 cells (Zhang et al., 2001). HEK 293 cells were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics (Invitrogen, Carlsbad, CA).

Transfection was performed using the lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Briefly, 1 day before transfection, HEK293 cells were subcultured in antibiotic-free medium supplemented with 10% fetal calf serum (Invitrogen, Carlsbad, CA). pcDNA3-TRPC4-HA and lipofectamine 2000 were mixed for 30 min before transfection. After transfection for 1 hr in OPTI-MEM I medium (Invitrogen, Carlsbad, CA), fresh antibiotic-free medium with 10% fetal calf serum was added.

2.5. Immunostaining of TRPC4 in BCEC

Fresh BCECs were peeled from fresh bovine corneas as described previously (Sun et al., 2000). Cultured BCECs were seeded onto coverslips for immunostaining. Fresh or cultured BCECs were washed with PBS three times. After fixation by 2% paraformaldehyde for 15 min, the coverslips were blocked by goat serum for 1 hr. Then anti-TRPC4 antibody (1:50) or the pre-immune IgG (1:50) was incubated with the coverslip for 1 hr. After washing by PBS-0.01% saponin three times of 15 min each, the secondary antibody against rabbit IgG conjugated with alexa fluor 488 (Molecular Probes) was incubated for another hour. The coverslips were further washed with PBS-0.01% saponin three times, 15 min each, and PBS twice, 10 min each. Cells were co-stained with 100 ng/ml 4',6'-diamidino-2-phenylindole hydrochloride (DAPI) to view the nuclei. Fluorescence was observed using a fluorescence microscope with a CCD camera (Nikon, Japan).

2.6. Synthesis and transfection of small interfering RNA

Two 21-nucleotide small interfering RNA (siRNA) sequences specifically targeting bovine TRPC4 were synthesized by the *Silencer*TM siRNA Construction Kit according to the manufacturer's instruction (Ambion, Austin, TX). Briefly, two oligonucleotide potentially targeting bovine TRPC4 was selected using the siRNA finder program at <http://www.ambion.com/>. The sense (siRNA1: AAGAAACAAAAGGGTTAAGCT-CCTGTCTC; siRNA2: AAGCTTTAACGTCTATGTTGGCCTGTCTC) and antisense oligonucleotides (siRNA1: AAAGCTTAACCCTTTTGTTCCTGTCTC; siRNA2: AACCAACATAGACGTTAAAGCCCTGTCTC) with T7 binding sequences were synthesized by Integrated DNA Technologies (Coralville, IA). Copies of sense and antisense single strand RNA were synthesized using T7 polymerase. These were combined and incubated overnight at 37°C to form double stranded RNA. Finally the synthesized siRNA was purified by a RNA binding column provided in the kit. Both siRNA oligonucleotides could knock down the expression of TRPC4 in cultured BCEC. By Western blotting, siRNA1 reduced the expression of TRPC4 by 69%, while siRNA2 reduced the expression by 42% (data not shown). We chose siRNA1 for all experiments shown in Figs. 4-8. We refer to siRNA1 as TRPC4-siRNA. A scrambled TRPC4 siRNA, which had the same composition of bases as TRPC4-siRNA, was also synthesized for use as a control. The sense and antisense oligonucleotides for synthesizing the scrambled siRNA were AATAGTACAATAGAGACAGTGCCTGTCTC, and AACACTGTCTCTATTGTACTACCTGTCTC, respectively. Primary BCECs were plated and grown in DMEM to 30% confluence before transfection. BCECs were transfected with 90 nM of each siRNA overnight using the Oligofectamine transfection reagent (Invitrogen, Carlsbad, CA). On the second day, fresh growth medium without antibiotics was added. On

the fourth day after transfection, cells were assayed by RT-PCR, WB, or Ca^{2+} measurements.

2.7. Measurements of intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$)

Intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) was measured with Fura-2, a calcium-sensitive fluorescent dye, as described previously (Xie et al., 2002). The cells on coverslips were loaded with Fura-2 by incubation in Ca^{2+} -rich Ringer containing $5 \mu\text{M}$ Fura-2/AM for 30 min at room temperature. Then cells were washed for 45 min in Ca^{2+} -rich Ringer without Fura-2. The coverslips were placed in a perfusion chamber designed for an inverted microscope (Diaphot; Nikon). Ca^{2+} measurements were done at room temperature as $[\text{Ca}^{2+}]_i$ could be monitored at $22 \pm 2^\circ\text{C}$ (Grynkiewicz et al., 1985) and we have routinely obtained consistent Ca^{2+} responses (Srinivas et al., 1998, 2002; Xie et al., 2002). Fura-2 was excited at 340 and 380 nm, whereas fluorescence emission was monitored at 505 nm using a microscope fluorimeter (Photon Technology International, Lawrenceville, NJ). Ca^{2+} measurements are shown as 340/380 nm ratios obtained from groups of 10–15 cells. All tracings are presented as smoothed (spline) plots of recorded data points using the SigmaPlot 2000 software (Chicago, IL).

2.8. Protocols for studying CCE and receptor-operated Ca^{2+} entry (ROC)

CCE was studied using the following protocol. Store depletion was achieved by application of $20 \mu\text{M}$ of the SERCA inhibitor cyclopiazonic acid (CPA) in Ca^{2+} -free solution. After allowing the $[\text{Ca}^{2+}]_i$ to peak and then decrease to a steady-state level, 1.4 mM Ca^{2+} was introduced. Ca^{2+} entry after Ca^{2+} add-back was CCE, assayed by the peak elevation of fluorescence ratios 340/380. The slopes of ratio 340/380 increase was measured as the rate of Ca^{2+} entry (Ratio (340/380)/sec).

Receptor activation can cause both CCE and ROC (Barritt, 1999; Berridge, 2000; Zitt et al., 2002; Shuttleworth, 2004). The purinergic agonist ATP ($100 \mu\text{M}$) was applied in Ca^{2+} -free solution. After allowing the $[\text{Ca}^{2+}]_i$ to peak and then decrease to a steady-state level, 1.4 mM Ca^{2+} was introduced. Ca^{2+} entry after Ca^{2+} add-back was a sum of CCE and ROC, assayed by the peak elevation of fluorescence ratios 340/380. The slopes of ratio 340/380 increase was measured as the rate of Ca^{2+} entry (Ratio (340/380)/sec).

Intracellular Ca^{2+} release, i.e. the Ca^{2+} store size, was assayed by the peak elevation of fluorescence ratios after application of ATP or CPA in Ca^{2+} -free medium. Student's *t* test was used to analyse the data; $p < 0.05$ was considered significant.

3. Results

3.1. Generation and characterization of the TRPC4 antibody

In order to examine the expression of TRPC4 in BCEC, we generated a rabbit polyclonal antibody (see Section 2) using a fusion protein as antigen. We PCR amplified the segment 755–975 aa of TRPC4 (Fig. 1A), and subcloned this region to the C-terminus of GST. Fig. 1B shows the induction of fusion protein GST-TRPC4c in bacteria, which is 50 kD (26 kD for GST + 24 kD for TRPC4c). The purified GST-TRPC4c was used to make the polyclonal

anti-TRPC4c antibody. Western blotting using the antibody detected the antigen (GST-TRPC4c), while the pre-immune IgG did not produce any signal (Fig. 1C), indicating that the anti-TRPC4c antibody specifically recognizes the antigen.

3.2. Detection of TRPC4 in BCEC by Western blotting and immunofluorescence

We then tested if the anti-TRPC4c antibody recognized heterologously expressed TRPC4-HA in HEK 293 cells. As expected, the antibody against HA recognized the TRPC4-HA protein (Fig. 2A, Lane 1), migrating at 100 kD in SDS-PAGE. The anti-TRPC4c antibody also detected the same band (Fig. 2A, Lane 2). The pre-immune IgG did not detect this band (Fig. 2A, Lane 5). To confirm that the antibody is specific to TRPC4, we did absorption experiments using the antigen (GST-TRPC4c) (Sun et al., 2000). In Western blotting using the anti-TRPC4c antibody incubated with the antigen (GST-TRPC4c, Fig. 2A, Lane 4), no band was detected, while the TRPC4-HA protein was still detected using the anti-TRPC4c antibody incubated with a control protein (GST, Fig. 2A, Lane 3). Western blotting using the T4nAb also detected the same band (data not shown). These results indicate that the anti-TRPC4c antibody is specific to the TRPC4 protein.

It is known that TRPC4 is expressed in the brain (Tang et al., 2000). We used bovine brain as a positive control. Fig. 2B shows that the anti-TRPC4c antibody recognized two bands migrating at around 80 and 75 kD, which are 15–20kD less than the expected from the full length protein (Fig. 2A). Western blotting using the T4nAb also detected the same bands (data not shown). Both bands could be absorbed by the antigen GST-TRPC4c, but not by GST (data not shown). The pre-immune IgG did not detect any bands (Lane 1). The anti-TRPC4c antibody detected only one band migrating at 80 kD for the membrane proteins of both fresh and cultured BCECs, while the pre-immune IgG did not detect any bands (Fig. 2C). Similarly, the 80 kD band could be absorbed by the antigen GST-TRPC4c, but not by GST (data not shown). Western blotting using the T4nAb also detected the same band size (data not shown). These results strongly suggest that TRPC4 protein is expressed in both fresh and cultured BCECs. The TRPC4 protein in native tissues (Fig. 2B and C) is smaller than the heterologously expressed protein (Fig. 2A). It is possible that the native protein is translated from a different starting site. It is also possible that the native proteins migrate aberrantly in the gel (Strubing et al., 2001).

Expression of TRPC4 was further examined using the immunostaining approach. While the pre-immune IgG did not stain the fresh BCEC (Fig. 3A), the anti-TRPC4c antibody significantly stained BCEC (Fig. 3B). Immunostaining of the cultured BCEC using the anti-TRPC4c antibody also gave similar results, while the pre-immune IgG did not stain (data not shown). Taken together, TRPC4 is expressed in both fresh and cultured BCEC.

3.3. RNAi knock-down of TRPC4 in BCEC

The small interference RNA technique (siRNA) is a newly developed approach to knock down the expression of a specific gene in native cells (Scherr et al., 2003). In order to evaluate the function of TRPC4 in BCEC, we utilized the siRNA approach to knock down the expression of TRPC4 and examined if the reduction of TRPC4 expression could affect Ca^{2+} signalling in BCEC.

Transfection of TRPC4-siRNA significantly reduced the expression of TRPC4 mRNA, compared with the scrambled siRNA transfected cells (Fig. 4A). At the protein level, we also observed a consistent reduction of TRPC4 in TRPC4-siRNA transfected cells relative to the scrambled siRNA control (Fig. 4B). These results show that siRNA can successfully decrease the expression of TRPC4 in cultured BCEC.

3.4. Effects of TRPC4-siRNA on Ca^{2+} signalling in BCEC

We and others have demonstrated both purinergic receptor activation of Ca^{2+} entry and CCE in BCEC previously (Srinivas et al., 1998, 2002; Cha et al., 2000; Xie et al., 2002). However, the molecular identity of receptor or store-operated Ca^{2+} entry is unclear in BCEC. Some studies suggested that TRPC4 is store-operated (Freichel et al., 2001; Philipp et al., 2000; Tiruppathi et al., 2002; Wang et al., 2004); but others supported a receptor-operated mechanism or ROC (Schaefer et al., 2000, 2002; Wu et al., 2002).

In order to examine the potential role of TRPC4 in Ca^{2+} signalling in BCEC, we first used the SERCA blocker CPA to evaluate the involvement of TRPC4 in CCE. After application of 20 μM CPA in Ca^{2+} -free solution, a Ca^{2+} peak was recorded (Fig. 5A, left peak), which is due to Ca^{2+} release from the intracellular store (Xie et al., 2002). The second peak after Ca^{2+} add-back is due to CCE (Fig. 5A). The traces of CCE in the TRPC4-siRNA and scrambled siRNA treated cells are similar (Fig. 5B). Fig. 6 shows summary data indicating that there was no difference in the Ca^{2+} release or Ca^{2+} add-back between the TRPC4-siRNA and the scrambled siRNA transfected cells. There was also no difference in the rate of Ca^{2+} entry between the TRPC4-siRNA and the scrambled siRNA transfected cells (data not shown). These experiments indicate that TRPC4-siRNA did not affect the Ca^{2+} store or CCE.

Since the above experiment indicated that TRPC4 was not involved in CCE, we next tested the potential role of TRPC4 in ROC in BCEC. After application of the purinergic agonist ATP (100 μM) in Ca^{2+} -free solution, a Ca^{2+} peak was recorded (Fig. 7A, left peak), which is due to Ca^{2+} release from the intracellular store. The second peak after Ca^{2+} add-back is due to both CCE and ROC (Fig. 7A; Barritt, 1999; Berridge, 2000; Zitt et al., 2002; Shuttleworth, 2004; Gill and Patterson, 2004). The Ca^{2+} release was similar, however, the second peak was significantly greater and faster in the TRPC4-siRNA than the scrambled siRNA treated cells (Fig. 7B). Fig. 8A shows summary data indicating that the Ca^{2+} release did not differ between the TRPC4-siRNA and scrambled siRNA treated cells, consistent with non-involvement of TRPC4 in Ca^{2+} release from the intracellular Ca^{2+} store. On the other hand, the amplitude and rate of Ca^{2+} entry from the bath were significantly bigger and faster in TRPC4-siRNA transfected cells relative to the scrambled siRNA transfected cells (Fig. 8B and C). Consistently, the area under curve 2 min after Ca^{2+} was added back in TRPC4-siRNA transfected cells was greater than the control ($p < 0.05$, not shown). Since TRPC4-siRNA knocked down the expression of TRPC4 significantly, these results suggest that TRPC4 may be a negative regulator of ROC, but not involved in CCE.

4. Discussion

In this study, we detected the expression of TRPC4 in fresh and cultured BCEC by RT-PCR, Western blotting, and immunostaining. RNAi knock-down of TRPC4 increased the Ca^{2+} entry level induced by the purinergic agonist ATP, but had no effects on CCE induced by the SERCA inhibitor CPA, suggesting that TRPC4 is involved in ROC, but not CCE.

We first developed a polyclonal anti-TRPC4c antibody by using the fusion protein approach. The anti-TRPC4c antibody specifically recognizes the antigen, heterologously expressed TRPC4, and native TRPC4 proteins (Figs. 1 and 2). Western blotting using another TRPC4 antibody (T4nAb) (Tang et al., 2000), which is against the N-terminal region of TRPC4, also detected a 80 kD TRPC4 in BCEC. Immunostaining also shows the expression of TRPC4 in BCEC (Fig. 3). The above results strongly support the expression of TRPC4 in both fresh and cultured BCEC. The siRNA technique is a newly developed approach to knock down the expression of a specific gene (Scherr et al., 2003). The TRPC4-siRNA successfully knocked down the expression of TRPC4 at both the mRNA and protein level (Fig. 4), indicating that the siRNA technique is effective in BCEC.

Although much effort has been put into the characterization of TRPC channels, the functions of TRPCs are still unclear (Clapham et al., 2001; Minke and Cook, 2002; Montell, 2001). However, it is clear that most TRPCs are downstream of PLC activation upon receptor stimulation. One of the main effects of purinergic receptor activation is store depletion by IP_3 . An important hypothesis is that TRPCs form part or all of the SOC pore. However, there is evidence for its role in CCE and also evidence against it. In particular, TRPC4 was thought to be a SOC channel (Freichel et al., 2001; Philipp et al., 2000; Tiruppathi et al., 2002; Wang et al., 2004) or involved in ROC (Schaefer et al., 2000; Wu et al., 2002). However, TRPC4 was also shown to be a dominant negative player in the channel complex of ROC (Schaefer et al., 2002).

In BCEC, ATP induced Ca^{2+} entry was increased in TRPC4-siRNA transfected cells compared with the scrambled siRNA control, while Ca^{2+} entry induced by store depletion through blocking SERCA by CPA, did not differ between the TRPC4-siRNA and scrambled siRNA-treated cells (Figs. 5-8). These results indicate that TRPC4 is not involved in CCE in BCEC, but is consistent with a role in negatively regulating ROC, similar to a previous report (Schaefer et al., 2002). Numerous second messengers such as IP_3 (Kuno and Gardner, 1987; Mozhayeva et al., 1991; Restrepo et al., 1990), IP_4 (Luckhoff and Clapham, 1992), arachidonic acid (Mignen and Shuttleworth, 2001; Mignen et al., 2001), diacylglycerol (Hofmann, 1999), protein kinase C (Venkatachalam et al., 2003) and the trimeric G proteins (Krautwurst et al., 1992) are downstream of receptor activation and may mediate or regulate ROC. To our knowledge, the components and molecular identity of ROC are not clear. We speculate that TRPC4 may interact with and inhibit ROC in BCEC. Future studies need to characterize if IP_3 , IP_4 , arachidonic acid, diacylglycerol, protein kinase C, G protein or other unknown factors can modulate the activation of ROC in BCEC.

In summary, we made an anti-TRPC4c antibody, which can specifically recognize the TRPC4 protein. TRPC4 was shown to be expressed in both fresh and cultured BCECs by

RT-PCR, WB, and immunostaining. The RNAi technique knocked down the expression of TRPC4 at the mRNA and protein level. TRPC4 may play a negative role in purinergic receptor induced Ca^{2+} entry, but not in CCE in BCEC. Further investigations are needed to reveal the molecular composition and mechanism of CCE and ROC in BCEC.

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Abbreviations

BCEC	bovine corneal endothelial cells
CCE	capacitative calcium entry
CPA	cyclopiazonic acid
ER	endoplasmic reticulum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPCR	G-protein coupled receptor
GST	glutathione S-transferase
IgG	immunoglobulin G
IP3	inositol trisphosphate
IP3R	IP3 receptor(s)
PBST	phosphate buffered saline with 0.1% Tween 20
PLC	phospholipase C
ROC	receptor operated Ca^{2+} entry

RT-PCR	reverse transcription-polymerase chain reaction
SERCA	sarco-endoplasmic reticulum Ca^{2+} -ATPase
SOC	store-operated Ca^{2+} channel
TRP	transient receptor potential
WB	Western blotting

Fig. 1.

Generation and characterization of the anti-TRPC4c antibody. (A) Domain structure of TRPC4. TRPC4 has ankyrin repeat (ANK), coiled coil (CC), and ion channel domains. The segment 755–975 aa was fused to the C-terminus of GST as an antigen to make the anti-TRPC4c antibody. The predicted size for GST-TRPC4c is 50 kD. (B) Coomassie staining of induced proteins GST and GST-TRPC4. (C) Western blotting using the anti-TRPC4c antibody to detect the antigen. Left, WB using the anti-TRPC4c antibody; right, WB using the pre-immune IgG.

Fig. 2.

Detection of TRPC4 in transfected cells, brain tissues and BCEC. (A) Western blotting using the anti-TRPC4c antibody to detect TRPC4 heterologously expressed in HEK293 cells. Lane 1, WB using the anti-HA antibody; 2, WB using the anti-TRPC4c antibody; 3, WB using the anti-TRPC4c antibody incubated with the GST protein; 4, WB using the anti-TRPC4c antibody incubated with the GST-TRPC4c protein; 5, WB using the pre-immune IgG. (B) Specific detection of TRPC4 protein in bovine brain membrane proteins by WB. Lane 1, WB using the pre-immune IgG; Lane 2, WB using the anti-TRPC4c antibody. (C) Specific detection of TRPC4 protein in BCEC by WB. Lane 1, WB of fresh BCEC membrane proteins using the pre-immune IgG; Lane 2, WB of cultured BCEC membrane proteins using the pre-immune IgG; Lane 3, WB of fresh BCEC membrane proteins using the anti-TRPC4c antibody; Lane 4, WB of cultured BCEC membrane proteins using the anti-TRPC4c antibody.

Fig. 3. Immunofluorescence staining of TRPC4 in fresh BCEC. (A) Immunostaining of fresh BCEC by the rabbit pre-immune IgG plus anti-rabbit IgG-conjugated alexa fluor 488. (B) Immunostaining of fresh BCEC by the rabbit anti-TRPC4c antibody plus anti-rabbit IgG-conjugated alexa fluor 488.

Fig. 4. siRNA technique knocked down the expression of endogenous TRPC4 in cultured BCEC. (A) RT-PCR analysis of the effects of TRPC4-siRNA on the expression of TRPC4 at the mRNA level, compared with the scrambled siRNA. GAPDH was used as a control. (B) WB analysis of the effects of TRPC4-siRNA on the expression of TRPC4 at the protein level, compared with the scrambled siRNA. β -actin was used as a loading control.

Fig. 5.

Representative tracings of Ca²⁺ release and CCE induced by the SERCA blocker CPA.

Cultured BCECs were transfected with either TRPC4-siRNA or scrambled siRNA (90 nM) for 4 days. Cells on coverslips were perfused as described in Section 2. (A) CPA (20 μ M) was applied in Ca²⁺-free solution. After 900 sec, 1.4 mM Ca²⁺ was added back to the perfusion. (B) Enlargement of the Ca²⁺ add-back traces.

Fig. 6.

Summary of the Ca²⁺ release (A) and entry (B) induced by CPA. (A) Comparison of Ca²⁺ release from the intracellular Ca²⁺ store by CPA in TRPC4-siRNA ($n=5$) and scrambled siRNA transfected cells ($n=5$), $p>0.05$. (B) Comparison of Ca²⁺ entry after store depletion by CPA in TRPC4-siRNA and scrambled siRNA transfected cells, $p>0.05$.

Fig. 7.

Representative tracings of Ca^{2+} release and CCE induced by the purinergic agonist ATP. Cultured BCECs were transfected with either TRPC4-siRNA or scrambled siRNA (90 nM) for 4 days. Cells on coverslips were perfused as described in Section 2. (A) ATP (100 μM) was applied in Ca^{2+} -free solution. After 700 sec, 1.4 mM Ca^{2+} was added back to the perfusion. (B) Enlargement of the Ca^{2+} add-back traces.

Fig. 8.

Summary of the Ca²⁺ release (A) and entry (B, C) induced by ATP. (A) Comparison of Ca²⁺ release from the intracellular Ca²⁺ store by ATP in TRPC4-siRNA ($n=5$) and scrambled siRNA transfected cells ($n=5$), $p>0.05$. (B) Comparison of Ca²⁺ entry after store depletion by ATP in TRPC4-siRNA and scrambled siRNA transfected cells. *, $p<0.05$. (C) Comparison of the rates of Ca²⁺ entry after store depletion by ATP in TRPC4-siRNA and scrambled siRNA transfected cells. *, $p<0.05$.